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13. ABSTRACT ***(Maximum 200 Words)***

It is believed that the primary function of the protein encoded by the NF-1 gene is as a negative regulator of the ras protooncogene. Mutation of the NF-1 gene therefore releases ras function from negative regulation leading to hyperplasia and defects in multiple neural crest derived tissues. We have embarked on the generation of conditional mutant mice at the NF-1 locus and at the ras-Gap locus since these two genes are thought to be the major negative modulators of ras activity. In the second, we have confirmed germline transmission and activity of the NF-1 conditional allele. In addition we have embarked on various cellular and cre-transgenic mediated strategies to further study NF-1 function as proposed. These results are presented. While we continue to encounter technical difficulties in the generation of the rasGap conditional mouse, studies related to ras signalling have been advanced in anticipation of the generation of the mouse strain.

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FOREWORD

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INTRODUCTION

Von Recklinghausen's neurofibromatosis type 1 (NF1) is a dominant autosomal disorder that strikes approximately 1 in 3500 individuals. The disease in humans results from the mutational inactivation of a single gene. It is characterized by anomalies of diverse cell types, many of which are of the neural crest lineage, including melanocytes and Schwann cells. The severity or penetrance of the different phenotypes can vary greatly between afflicted individuals. However, the most serious life-threatening health situation occurs when neurofibromas/Schwannomas progress into malignant neurofibrosarcomas. The *NF1* gene product, neurofibromin, is a large cytoplasmic protein of over 2,800 amino acids that exhibits structural and functional homology to the GTPase-activating protein (GAP), both of which biochemically function as negative regulators of GTP-bound Ras proteins. The prevailing theory on the mechanism of Neurofibromatosis type 1 is that inactivation of the *NF1* gene results in elevated Ras-GTP levels which causes excessive signaling to downstream effector molecules in the mutant cells. Thus, over-stimulation of signal transduction pathways caused by uncontrolled Ras-GTP levels is thought to play a major role in the development of this disease. Furthermore, oncogenic mutations in *ras* genes that encode dominant constitutively active GTP-bound forms of Ras are frequently observed in a diverse range of human malignancies. Therefore, while it is clear that study of the *NF1* gene is essential to the understanding of this particular disease, the analysis of other proteins that regulate Ras activity in the same way as neurofibromin also merit careful study.

This proposal concerns the development and analysis of mouse models for the study of Neurofibromatosis. In past years, we have isolated the mouse equivalent *NF1* gene and inactivated it by mutation, allowing us to study the function of neurofibromin in great detail [1]. For example, our studies have revealed that the *NF1* gene may have important functions in regulating how neurons survive and in the interplay between neurons and non-neuronal cells [3]. Others have used this mutation to study the effects of *NF1* loss on lymphomas [4]. Our studies of *GAP* mutant mice have also revealed defects in blood vessel formation and in the survival of neuronal cells [2]. Moreover, combination of the *NF1* and *GAP* mutations in mice has revealed genetic interactions during both embryonic development and in tumorigenesis [2] (Henkemeyer et al., manuscript in preparation). The availability of these mouse models has provided important data, but suffers from the drawback that both *NF1* and *GAP* homozygous mutant animals perish during fetal development, thus limiting our analysis to the early phases of embryonic development. In this application, we proposed to generate improved mouse models that will permit analysis of more mature tissues and adult animals in attempts to more closely mimic the human condition. This strategy will permit us to inactivate the *NF1* and *GAP* gene products either separately or in combination in selected tissues, cell types, and at specified times of the animal's life. These animal models will provide powerful tools for understanding the function of the *NF1* gene and the consequences of its inactivation in the regulation of Ras signaling in the cell. Moreover, continued analysis of the genetic interactions of *NF1* and *GAP* should lead to a greater understanding of the biological functions of these Ras GAPs and may help define their role in the development of diseases including Neurofibromatosis type 1. These studies may help in the development of novel strategies to treat Neurofibromatosis related diseases.

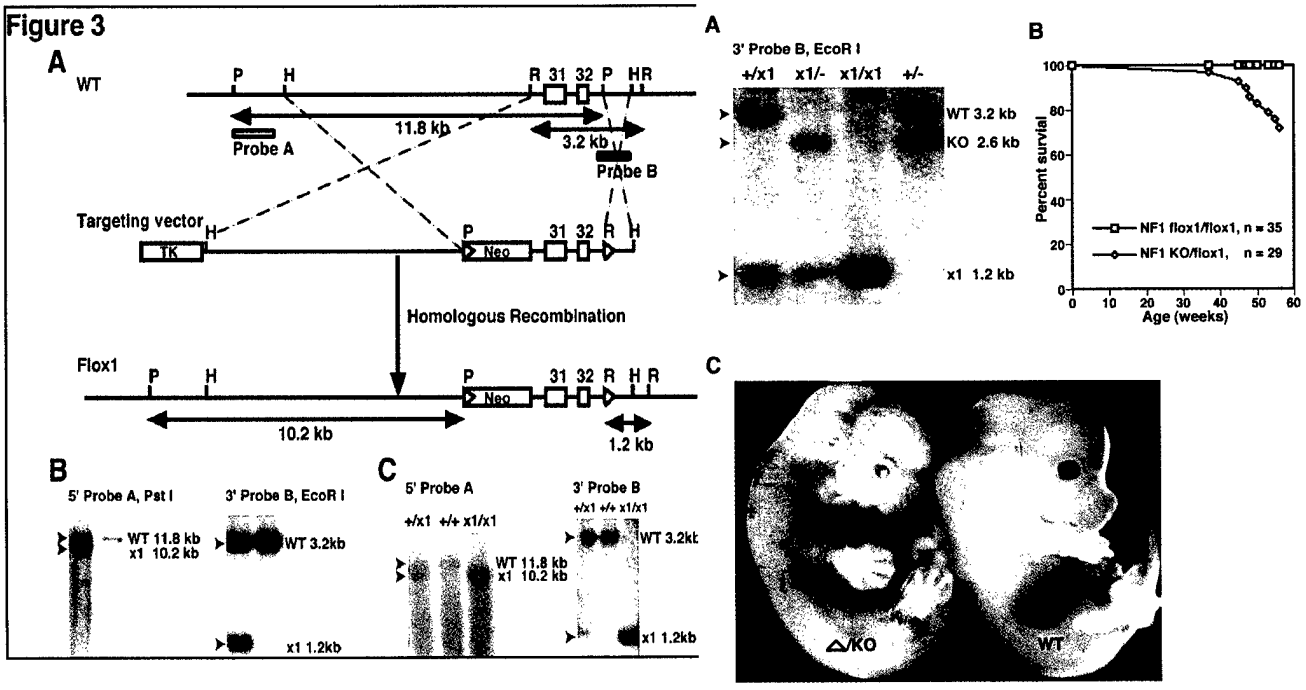
This application brings together expertise, background and reagents to study Neurofibromin, GAP and *ras* regulation. Our proposal concerns the establishment of recent advances in gene knockout technology that will permit us to study the consequence of mutations in the *NF1* and *GAP* genes beyond the presently available embryonic stages. We proposed to generate conditional mutant mice through the implementation of a site specific recombinase (Cre) to catalyze recombination when expressed in cells that contain the unique target (*loxP*) sequences. We proposed five Objectives or Specific Aims. Objective 1 extends ongoing studies on *NF1* mutations. Objective 2 proposed to employ identical strategies to that of Objective 1 to generate and obtain germline transmission of a conditional mutation in the *GAP* gene.

The primary goal of this award has been to examine the role of Nf1 and p120Gap embryonic development as well as their interaction in Nf-1 disease. This work has moved forward at an accelerated pace in some areas and due to technical limitations at a relatively slower pace in others. The components of the proposal that rested on the generation and analysis of a conditional mutation at the Nf-1 gene have moved rapidly and already revealed a rich source of information. Below, I describe the progress in objective 1 as related to tasks 1-6. A hindrance in a component of the proposed work has been the difficulty in generating a conditional mutant of the p120Gap. Thus many of the objectives or on hold until this mutation is transmitted to the germ line. However, in lieu of the p120Gap mutation, Dr. Henkemeyer has made considerable progress (as outlined) in the understanding ofp120Gap signalling inside the cell and this progress will be very instructive in our analysis of subsequent experiments.

Objective 1. Tasks 1-2.

Our previous experience with the generation of a null mutation has confirmed that exon 31 is essential for NF1 function (Brannan et al., 1994; Vogel et al., 1995). Figure 3A indicates the strategy we employed to place *lox* sites flanking exons 31 and 32. PCR primers encoding the *lox* sequences (34bp) plus an Eco R1 restriction enzyme - site that are designed for diagnostic purposes (18 bp) - were inserted into introns that flank exons 31 and 32 of the NF1 gene. In this construct, the neo selectable marker lies within the *lox* sites such that after *cre* mediated recombination, the neo gene is removed together with exons 31 and 32. ES cells were next transfected with the target vector and homologous recombinant cells were screened, clonally purified and injected into blastocysts for the formation of germline chimeric mice. Germline transmission was obtained and heterozygous (Figure 3B) and homozygous (Figure 3C) mice were viable as indicated by Southern blots employing probes external to the recombination

Figure 4



cassette. We next examined the viability of the recombinant (**NF1flox**) allele. This was done by crossing the conditional allele into the null background to generate compound heterozygous mice that had a null allele on one chromosome and the NF1flox allele on the second chromosome (Figure 4A; x1/-). As shown in Figure 4B, the **NF1flox/-** compound heterozygous mice behave indistinguishably from NF1+/- heterozygotes and the **flox/flox** homozygotes are indistinguishable from wildtype mice. As previously demonstrated, NF1+/- mice begin to die around 11 months (Jacks et al., 1994). Thus by survival criteria, the NF1flox allele appears indistinguishable from the wild type allele in spite of harboring to flox sites and a neo gene within its intronic sequences.

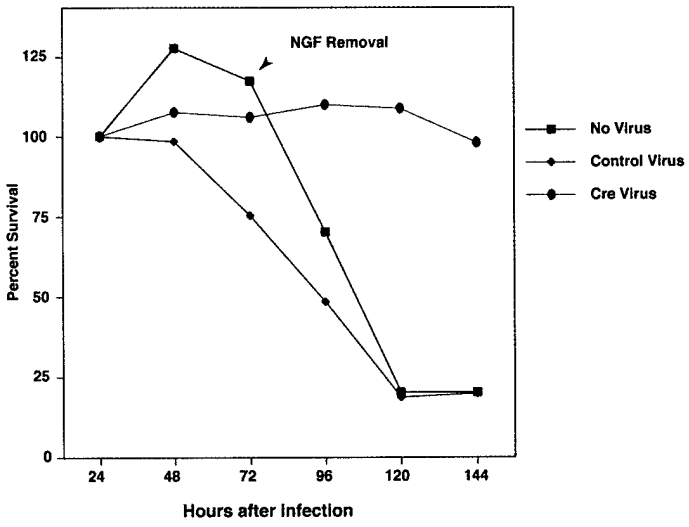
The original NF1-/- null embryos perish at E13.5 and are characterized by edema, abnormal heart, vascular malfunction and reduced pigmentation of the retina (Brannan et al., 1994; Jacks et al., 1994; Vogel and Parada, 1998). We have crossed the NF1flox/- compound heterozygote to a transgenic Cre expressing mouse line that expresses the cre recombinase in the germline. Thus effective ablation of the NF1flox allele in the germline should result in a phenocopy of the original NF1-/- embryos. As shown in Figure 4C, NF1flox/-;TG-cre mice are indistinguishable from NF1-/- embryos. This demonstrates that *in vivo*, the NF1flox allele is readily recombined to make a null allele through coexpression of cre recombinase.

These results indicate the successful generation of a conditional mutation of the NF1 gene. The entirety of the present proposal will center on exploitation of this functional model system to explore NF1 function in vivo and in primary culture.

Tasks 3-4. Primary culture studies.

DRG neurons: In the previous award period, we demonstrated that DRG neurons lacking NF-1 function were liberated of neurotrophin requirement for survival (Vogel et al., 1995). Our interpretation of the data was that removal of NF1 activated the ras pathway even in the absence of neurotrophins thus rendering the neurons neurotrophin independent. This interpretation, though consistent with all the data, could not rule out the formal possibility that the loss of neurotrophin requirement by sensory neurons might not be the consequence of earlier developmental effects (perhaps in the neural crest) which could lead to the formation of neurotrophin independent neurons.

Figure 6



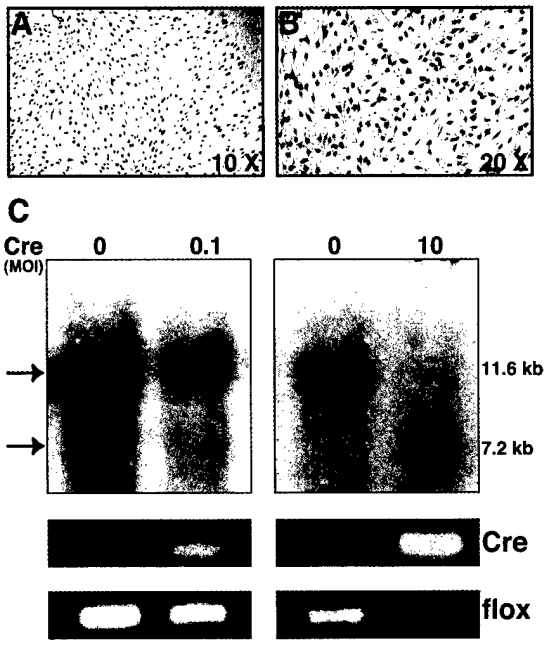
With the availability of the NF1flox mice we have finally been able to address this issue directly. As we have previously shown, DRG neurons can be cultured directly from mice and be exposed and productively infected by adenoviruses (Klesse and Parada, 1998). We have used adenoviral technology, which is well established in my laboratory (Klesse et al., 1999; Klesse and Parada, 1998), to produce a CMV-Cre adenovirus. NF1flox/- compound

heterozygous embryonic DRG neurons to cre recombinase can be scored by PCR (not shown). At MOI=20, virtually all DRG neurons in the cultures show deletion of the conditional allele.

Thus, DRG cultures can be effectively manipulated to ablate NF1 for cell biological and biochemical experiments. As indicated in Figure 6, neurons were cultured from NF1flox/- mice and subjected either to mock infection (no virus), infection with a CMV-lacZ expressing virus (Control virus), or with the CMV-cre virus. After neurotrophin removal, cultures were monitored for survival. Only the cultures exposed to Cre recombinase (and thus rendered NF-1 deficient) survived in the absence of neurotrophins. These data provide firm evidence that the action of NF-1 is directly on neurotrophin signalling. Specific Aim 1 will directly address and extend these studies to additional neuronal subtypes and to later times in development.

Schwann cells: Schwann cells or their precursors are likely the originators of neurofibromas and/or neurofibrosarcomas (Sherman, 1998). Ratner and colleagues have made elegant studies to examine the properties of embryonic NF-1-/-Schwann cells (Bhattacharyya et al., 1994; Kim et al., 1997; Kim et al., 1995; Sherman, 1998). However these studies have been hampered by the limitation that these cells can only be produced from a limited window of opportunity (before E13.5) and the quantities that are obtained are limited. We have cultured adult sciatic nerve Schwann cells from the NF1flox mice using standard culture procedures provided us by Mary Bunge. We are able to collect the sciatic nerves of a large number of mice at once and thus can prepare large quantities of cells. As shown in Figure 7, these cells grow robustly in the presence of forskolin and glial growth factor (GGF). Furthermore, these cells can be efficiently infected with adenoviruses. As shown in panels A & B, greater than 90% of the cells stain for Lac Z. Figure 7C shows Southern blot (upper) and PCR based (lower) assays for reduction of the gene by coexposure to the cre-expressing adenovirus. MOI =10 is sufficient to ablate NF-1 from the majority of cells.

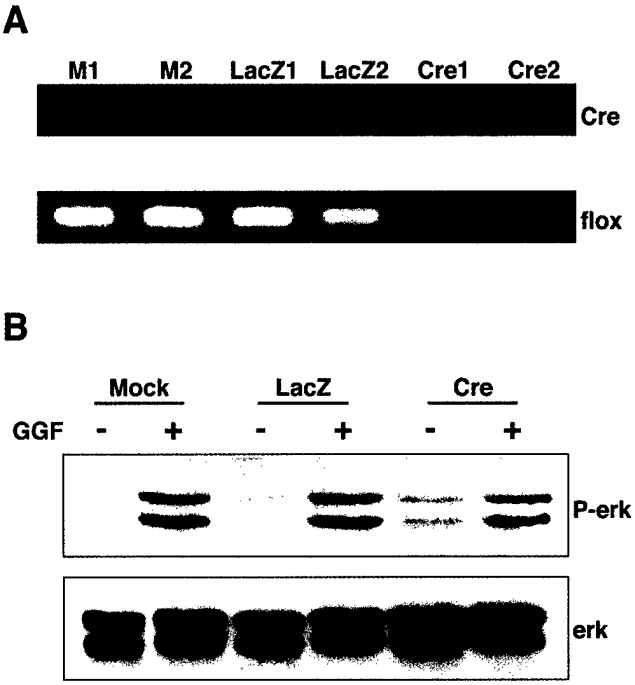
Figure 7



The ability to grow large numbers of cells will afford us the possibility of performing assays that are otherwise impractical with the original null NF-1 embryos. As shown in Figure 8, Nf1flox Schwann cells were either mock infected (M1 & M2), or exposed to LacZ or Cre expressing adenoviruses. Panel A shows that only the Cre infected cells recombined the floxed genes. These Schwann cell cultures were maintained in the presence or absence of GGF. As shown in Panel B, phopho-erk antibodies detect activated erk only in the GGF cultured cells except for the Cre recombined cells. In this latter case, erk is found active in the cultures that do not have GGF. These data demonstrate that downstream effectors of the ras pathway are constitutively activated in Schwann cells when NF1 function is removed.

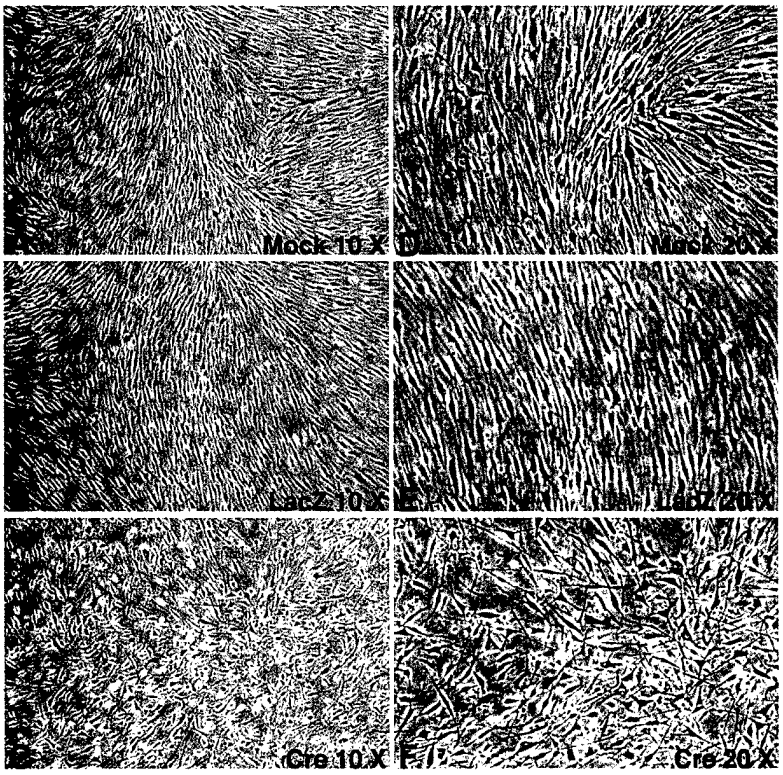
Cultured Schwann cells have a distinct morphology characterized by alignment into organized swirls (Figure 9 A-E). However, upon exposure to cre recombinase and ablation of NF-1 function, these cells acquire a distinct morphological change. As seen in Figure 9 C & D, elimination of NF-1 function causes the cells to become refractile and disorganized. By classical oncogenic criteria these cells have lost contact inhibition and appear morphologically transformed (Parada et al., 1984; Parada et al., 1982; Parada and Weinberg, 1983).

Figure 8



Promoter specific Cre Adenoviruses: As described in detail below, tissue specific promoters will be employed to drive Cre transgene expression for the purpose of inactivating NF-1 in specific tissue compartments. An additional approach toward spatial and temporal inactivation of NF-1 is through the application of Adenoviruses to cultures as described above, or *in vivo*. We have therefore undertaken to generate recombinant adenoviruses that express cre under neuronal or glial specific promoters. These viruses will be used to inactivate NF-1 regionally in the NF-1flox mouse.

Figure 9



3) Synapsin I-cre transgenic mice. The Synapsin I gene is expressed exclusively in neurons (Schoch et al., 1996). Our collaborator at UCSD, Dr. Jamey Marth, has provided us with two independent lines of cre-expressing transgenic mice under the control of the Synapsin I promoter (Syn-cre mice).

ß-actin/LacZ reporter mouse. While the description of tissue specific promoters has been in the literature for many years, it is evident that the use of such promoters to drive transgene expression, are not always successful. Introduction

of transgenes into the genome can result in modulatory expression effects. The neighboring chromatin can sometimes exert control over the introduced promoter and cause ectopic, circumscribed, or diminished expression. It is therefore important to characterize each transgenic founder to determine the precise expression in that mouse and its derivatives. Therefore, to ascertain the spatial and temporal expression of cre in the transgenic mice to be used in this proposal, we employ a reporter mouse strain that harbors a beta-actin driven LacZ gene that requires cre function for activation of the lacZ gene (Tsien et al., 1996). Thus only tissues where cre has been expressed stain for LacZ. Figure 10 shows four panels of mouse embryos stained for lacZ. The Syn-cre transgenic (upper left) and the beta-actin reporter transgenic (upper right) do not stain blue. However, upon crossing these mice, the resultant progeny _-actinLacZ/Syncre) show _-gal activity in tissues that normally express the Synapsin I gene, namely in differentiated neurons as evidenced by expression in the head and spinal cord region. Figure 11 shows vibratome sections of the Syn-cre/lacZ embryos. Figure 11, Panel A illustrates the absence of staining in E12.5 ventricular regions where undifferentiated neuronal progenitors are in the process of dividing. LacZ staining is limited to the peripheral regions of the embryonic brain where maturation and differentiation is taking place. Panel B shows LacZ expression in the spinal cord and lightly in DRG. A sagittal view of the thoracic region of an E15.5 embryo illustrates the cranio-caudal nature of embryonic development with the more mature cranial DRG expressing the strongest LacZ in a gradient toward the less differentiated, lighter staining ganglia more caudal.

Figure 10

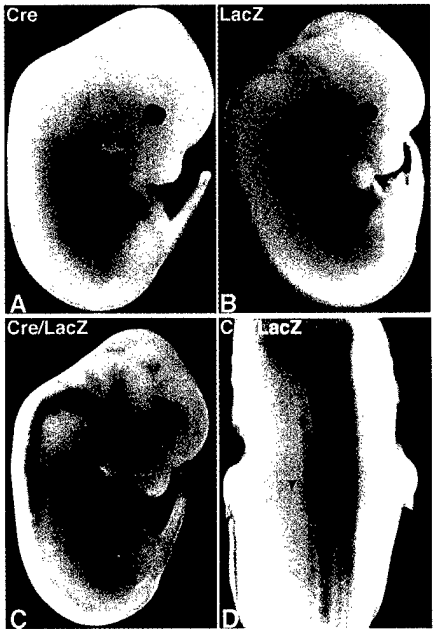
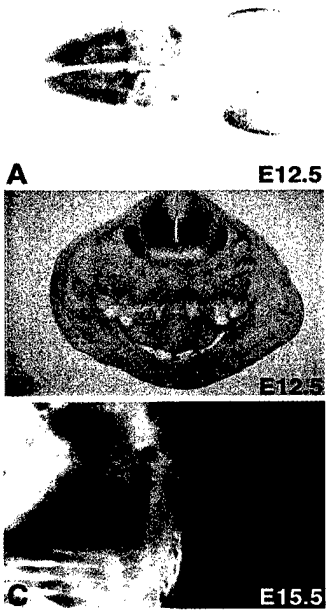


Figure 11



To further examine the specificity of Cre expression in the Syn-cre mouse, we examined histological sections of the CNS using immunohistochemistry with LacZ and neuron specific monoclonal (NeuN) antibodies (Chemicon International Inc.; MAB377). Figure 12 shows

immunofluorescence of the neo-cortex (A-F) and spinal cord (G-I) of Syn-cre/LacZ newborn pups. Red fluorescence is a neuron specific staining and Green fluorescence is the LacZ specific antibody (note that we observe some non-specific background staining with this

Figure 12

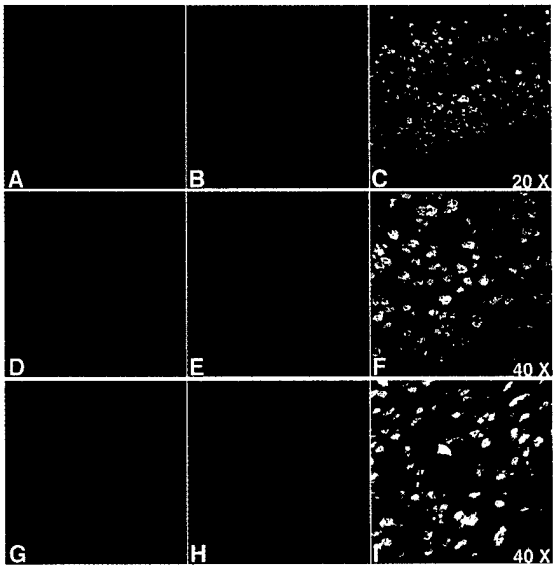
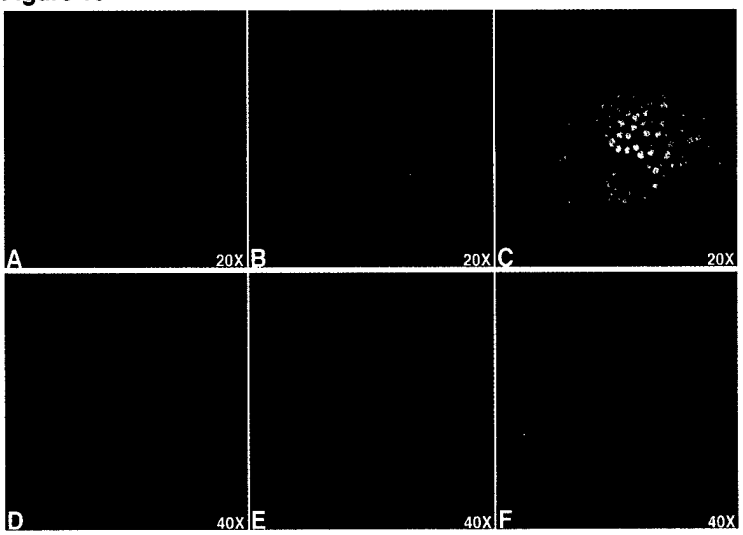


Figure 13



antibody). The right panels show double labelling in yellow with very accurate colabelling of the neuron specific and LacZ antibodies indicating cre activity specifically in neurons. Figure 13 examines coexpression of LacZ and neuron specific markers in the hippocampus (A-C) and cerebellum (D-F). In CA1-CA3 boundary of the hippocampus it is again clear that the LacZ activity is confined to neurons. Our NeuN monoclonal antibody is curiously not expressed in Purkinje cells (see manufacturer; Figure 13D). Thus, only the granule cell layer fluoresces red. The synapsin promoter however induced cre expression (and thus LacZ) in both Purkinje cells and in granule cells (Figure 4E). Thus, colabelling is confined to the granule layer even though lacZ staining remains evident in Purkinje cells.

Objective 2 (Henkemeyer laboratory) is listed as Task 7 in the statement of work in our original application. The main goal of this Objective is to generate a conditional null mutation in the mouse *GAP* gene by flanking the two exons encoding amino acids 172 to 267 with Cre recombinase specific *loxP* sequences. At the end of year 1 we reported to have successfully generated the conditional *Gap* targeting vector and, out of 2,883 embryonic stem (ES) cell lines screened, identified only 2 lines that exhibited homologous recombination at the *Gap* locus, designated VIII-22 and V-40. This unexpected result is in stark contrast to what we previously observed using the pMGAP.G7.7 targeting vector to obtain the null allele where 8 of 108 ES cell lines isolated exhibited homologous recombination [Henkemeyer, 1995 #4]. This greatly reduced frequency could be due to the presence of the repeated *loxP* and *frt* sequences in the new p*GAP-loxP-neo* vector, which may inhibit homologous recombination events. We performed Southern blot analysis of purified genomic DNA from the VIII-22 and V-40 ES cell lines to characterize the homologous recombination into the *GAP* gene. Unfortunately, we determined that only one of these 2 cell lines (V-40) had incorporated both desired *loxP* sequences into the *GAP* gene. Over the past year we have generated a large number of chimeric mice with the *Gap* V-40 ES cell line. However, quite unfortunately, none of the chimeric mice have transmitted the mutation through the germline. Given the key importance of having to obtain germline transmission of a conditional *Gap* mutation, we have attempted to obtain additional conditional *Gap* ES cell lines in the hopes that a different cell line will be able to contribute to the germline. Unfortunately, given the extremely low rate of homologous recombination observed, we have not yet obtained a new ES cell line targeted with our original conditional *Gap* plasmid vector. To increase our chances of recovering a properly targeted conditional *Gap* mutation, we have resorted to constructing a new *Gap* targeting vector and have begun electroporating this into ES cells in attempts to achieve a higher frequency of homologous recombination (see below).

In addition to the V-40 ES cell line that has failed to go germline, we also recovered a sub-optimal *Gap* targeted ES cell line (VIII-22) that has only one *loxP* sequence. Although this insertion did not generate a conditional allele as it has just one *loxP* site, we thought it might still provide useful information as the insertion of the *frt*-flanked *neo* cassette in the *GAP* intron may have created a hypomorphic allele. Therefore, chimeric mice were generated with this ES cell line and germline transmission was obtained. To determine whether the inserted *neo* cassette affected the function of the *Gap* gene, heterozygous male and female mice harboring this potential hypomorphic mutation were mated to generate homozygotes. Through this analysis it was found that homozygotes survived to adult stages and appeared normal. Given that homozygotes for the protein-null *Gap* allele die in utero at embryonic day 9.5 (E9.5; Henkemeyer et al., 1995), these results indicate that insertion of the *neo* cassette has little, if any, consequence to the normal function of the *Gap* gene.

The original conditional *Gap* targeting vector was engineered with the *neo* cassette flanked by *frt* sequences to allow removal of this cassette by expression of the Flp recombinase. Since the data described in the above paragraph demonstrated that the presence of the *neo* cassette did not appear to interfere with normal *Gap* function, we have reconstructed a conditional *Gap* targeting vector whereby the *neo* cassette is not flanked by the *frt* sequences. Like the original conditional vector, this new vector still contains a pair of *loxP* sequences flanking the two exons encoding GAP amino acids 172 to 267. Thus, we hope that by eliminating the repeated *frt* sequences, a much greater frequency of homologous recombination will be obtained. This new vector is presently being electroporated into ES cells. We expect this new vector will allow us to obtain germline transmission of the conditional mutation. Once germline transmission is achieved, we will be able to selectively inactivate *Gap* gene function following expression of Cre recombinase in desired cell/tissue types. When deleted by Cre recombinase, the disruption of these two exons will lead to a protein null mutation of GAP.

Additional studies of GAP and neurofibromin function

While we have worked on generating the conditional *Gap* mutation in mice, we have continued to study in detail the phenotypes associated with our original null mutation (Henkemeyer et al., 1995). In collaboration with Dr. David Gutmann, we have analyzed *Gap*/+ heterozygous adult mice to determine whether there is an increase in astrocyte proliferation. While these studies have shown that *Nf1*/+ heterozygous brains do show some level of increased astrocyte proliferation, the *Gap*/+ heterozygotes appeared normal (Gutmann et al., 1999). Thus, astrocyte growth rates appear to be particularly sensitive to the levels of neurofibromin protein. The specific effect that decreased levels of neurofibromin expression in heterozygous mice can lead to increased astrocyte proliferation may provide a molecular explanation for the development of astrocytic growth abnormalities observed in human patients with NF1.

A major series of experiments being performed in the Henkemeyer lab involve studying a novel genetic interaction that has been observed in developing mouse embryos by combining the null mutation in *Gap* with a targeted mutation in the gene encoding the SH2 containing tyrosine phosphatase, Shp2. Dr. Henkemeyer played a major role in the generation and analysis of both the *Gap* and *Shp2* mutations (Henkemeyer et al., 1995; Saxton et al., 1997). Both GAP and Shp2 proteins contain SH2 domains and can, therefore, bind with high affinity to other tyrosine phosphorylated proteins, including activated receptor tyrosine kinases. Like *Nf1* and *Gap*, *Shp2* is an ancient gene and its Drosophila homolog (called *corkscrew*) has been implicated to function in a number of developmental pathways, including early embryonic patterning and photoreceptor cell differentiation. Both GAP and Shp2 are implicated in regulating Ras-GTP levels downstream of receptor tyrosine kinases. GAP is a Ras GTPase Activating Protein which functions to greatly accelerate the hydrolysis of GTP to GDP, thus leading to Ras inactivation. Shp2 biochemical properties are less well understood, however this protein appears to lead to the activation of Ras and MAP kinase signaling. These two proteins are essential for normal development, as mice homozygous for a targeted mutation in either *Gap* (**Figure 1**) or *Shp2* (**Figure 2**) are embryonic lethal by E10. Moreover, Shp2 and GAP appear to be functioning in the same types of cells in the embryo as each of the single mutants exhibits phenotypes in posterior elongation, the notochord and vascular organization. Moreover, the *Shp2* mutant phenotypes closely resemble those observed following deletion of the Fibroblast growth factor receptor 1 (FGFR1; Yamaguchi et al., 1994; Deng et al., 1994) or one of its ligands, FGF8 (Sun et al., 1999). We formulated a hypothesis that upon binding of FGF8, the activated FGFR1 transduces a signal into the cell and that these signals are regulated in a negative fashion by GAP and in a positive fashion by Shp2 (**Figure 3**).

To test this hypothesis, we carried out a series of genetic experiments to determine whether the elimination of both a positive (Shp2) and negative (GAP) regulator of Ras from the same embryo (in double mutants) would suppress the phenotypes associated with the individual single mutants (Cowan, Yokoyama and Henkemeyer, manuscript in preparation). We focused our analysis between E8.5 to E10.5 utilizing two markers for early embryonic development, one for axial mesoderm (node and notochord) and the other for endothelial cells. As judged by overall morphological appearance and the expression of the beta-gal markers, the vast majority (84%) of *Shp2+Gap* double mutants examined at E9.5 exhibited full suppression of the respective single mutant phenotypes (**Figure 4 and Table 1**). Double mutants lacking Shp2 and neurofibromin did not suppress the *Shp2* mutant phenotypes, demonstrating the specificity of the genetic interaction with *Gap* (**Table 2**). Furthermore, we performed biochemical analysis of *Gap* and *Shp2* mutant fibroblasts and determined that cells lacking GAP show elevated levels of active MAP kinase following exposure to FGF, while cells lacking Shp2 show reduced levels of MAP kinase after stimulation with FGF (**Figure 5**). These results suggest that Shp2 and GAP work in concert to control Ras-GTP levels downstream of receptor tyrosine kinases, and that proper regulation of these signals are crucial for early mammalian development.

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Table 1. Generation of GAP+Shp2 double mutants

Gap/+;Shp2/+ ♂ X *Gap/+;Shp2* ♀

↓

1 of 16 are double homozygous

Results from E9.5 collections

Gap	*Shp2*	# progeny	Phenotype
+/+	+/+	6	WT
+/-	+/+	14	WT
+/+	+/-	29	WT
+/-	+/-	68	WT
-/-	+/+	8	GAP
-/-	+/-	18	GAP
+/+	-/-	11	Shp2
+/-	-/-	25	Shp2
-/-	-/-	12	10 WT, 2 Shp2

Table 2. Generation of *Nf1+Shp2* double mutants

Nf1/+;Shp2/+ ♂ X *Nf1/+;Shp2* ♀

↓

1 of 16 are double homozygous

Results from E9.5 collections

Nf1	*Shp2*	# progeny	Phenotype
+/+	+/+	16	WT
+/-	+/+	20	WT
+/+	+/-	25	WT
+/-	+/-	75	WT
-/-	+/+	19	WT (*)
-/-	+/-	33	WT (*)
+/+	-/-	15	Shp2
+/-	-/-	28	Shp2
-/-	-/-	10	Shp2

(*) Nf1 mutant phenotype is not apparent until E12.

Figure legends DOD.Year2 report

Figure 1. Whole-mount of a *Gap* single mutant collected at E9.5 and stained with the beta-gal marker *cordon-bleu* to label the notochord and ventral floorplate of the neural tube. The mutant exhibits the typical *Gap* phenotype of arrested posterior elongation, kinked neural tube and defective cardio-vascular development as indicated by the enlarged pericardium.

Figure 2. Whole-mount of seven different *Shp2* single mutants and one wild-type littermate collected at E9.5 and stained with the beta-gal marker *cordon-bleu*. Mutants exhibit the typical *Shp2* phenotype of severe defects in gastrulation as evident by disruptions in the notochord and anterior-posterior patterning.

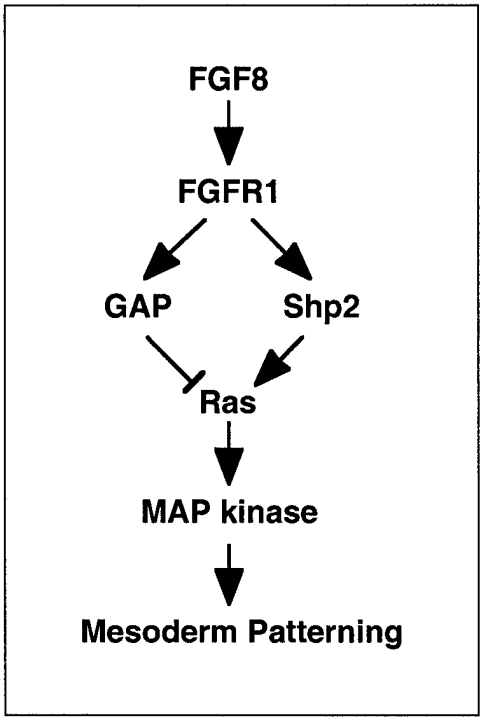

Figure 3. Hypothesized biochemical pathway whereby GAP and Shp2 regulate downstream signaling initiated by FGF8 and FGFR1.

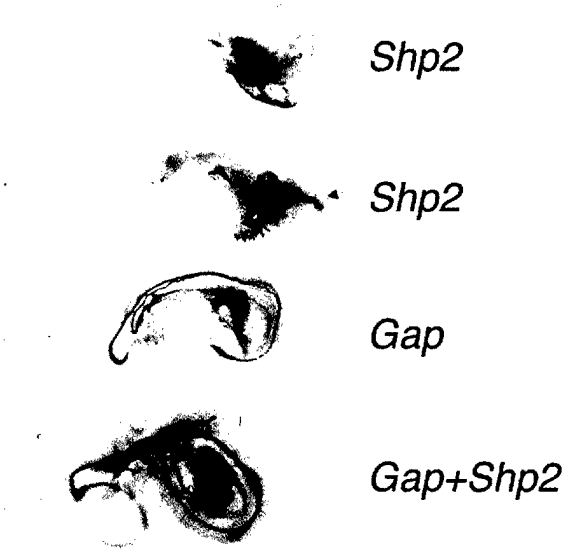

Figure 4. Reciprocal suppression of *Gap* and *Shp2* single mutant phenotypes in *Gap+Shp2* double mutant embryos. Compound heterozygous *Gap/+;Shp2/+* males and females were mated and embryos at E9.5 were collected and stained whole-mount for the *cordon-bleu* beta-gal marker. Note the complete suppression of the notochord defects observed in the individual single mutants.

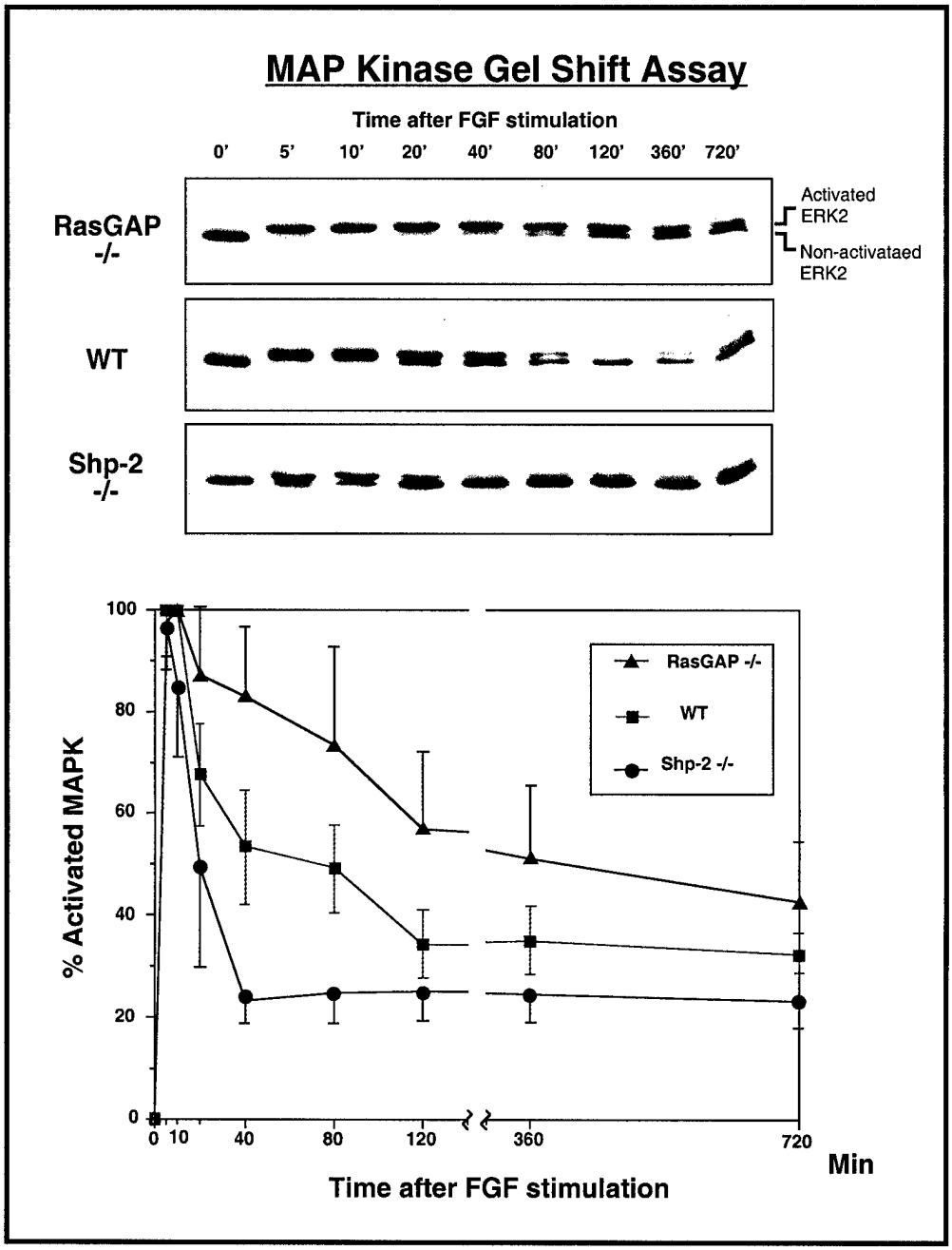

Figure 5. MAP kinase activation in wild-type (green), Gap single mutant (red) and Shp2 single mutant (blue) fibroblast cell lines.

CONCLUSIONS

Our progress in the second year has been considerable if somewhat uneven. The lack of current success in generating the conditional allele for rasGap has precluded some key and interesting experiments. However in the meantime, our progress with the study of NF-1 and its role in development and cancer is proceeding very well. In addition, the availability of the various proven cre transgenic and adenoviruses generated in this first period mean that as the Gap conditional mutants come on line, we will be in a position to move those experiments and the double mutant experiments rapidly.